

Spanish Patent No. 2 020 148 A6

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Job No.: 345-83033

Ref.: 89025515

Translated from Spanish by the Ralph McElroy Translation Company  
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INDUSTRIAL PROPERTY  
REGISTRY  
PATENT NO. 2 020 148 A6

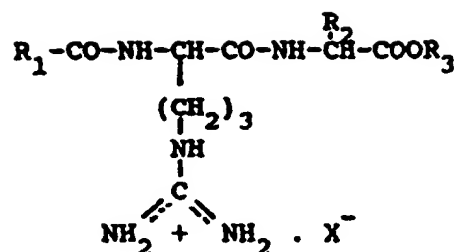
Int. Cl. <sup>5</sup> :	C 07 K 5/06 //A61K 7/50
Filing No.:	9001578
Filing Date:	June 7, 1990
Date of Announcement of the Granting:	July 16, 1991
Publication Date of the Patent:	July 16, 1991

PROCEDURE FOR THE SYNTHESIS OF DIPEPTIDES OF FATTY CHAIN N- $\alpha$ -ACYL  
ARGININE AND PURE AMINO ACIDS AS ANTIMICROBIAL IONIC SURFACTANTS.

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Abstract

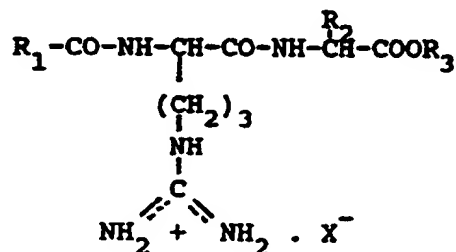
The procedure for the synthesis of dipeptides of fatty chain N- $\alpha$ -acyl arginine and pure amino acids as antimicrobial ionic surfactants is related to the preparation of lipopeptidic surfactants in which the hydrophilic part is formed by a dipeptide whose amino acid carrying the carbonyl group to the peptide bond is arginine, and the hydrophobic part consists of a fatty acid bonded to the arginine free amino group through an acyl bond according to the scheme of the formula.



These dipeptides are applicable in multiple industrial areas: cosmetics, dermopharmacy, foods, biotechnology, etc.

### Description

The present invention relates to the synthesis of antimicrobial lipopeptidic surfactants and, more particularly, to a family of N- $\alpha$ '-acyl dipeptides in which the hydrophilic part is formed by a dipeptide whose amino acid that carries the carbonyl group to the peptide bond is arginine, and the hydrophobic part consists of a fatty acid bonded to the arginine free  $\alpha$ -amino group by an acyl bond according to scheme [I]



These surfactants can be cationic or amphoteric and in either case they present excellent antimicrobial properties and are not irritants. Furthermore, they are capable of forming micelles, liquid crystals, emulsions, and microemulsions whose technology is applicable in multiple industrial areas: cosmetics, dermopharmacy, foods, biotechnology, etc.

It is well known that materials such as cosmetics, dermapharmaceutical products, foods, etc., are easily attacked by microorganisms, decomposing over time if they are not adequately protected.

Different antifungals or antiseptics exist for these purposes. However, these compounds either do not totally inhibit microbial growth, or have the drawback of irritability or toxicity. Among the investigations leading to non-irritant antiseptic or antifungal agents, surfactants derived from protein material have roused great interest, especially in recent years, not only because of the simplicity of their structure but because of the multifunctionality that they present.

Until now the majority of published works and registered patents about surfactants derived from amino acids and/or peptides with antimicrobial activity have focused on the study and application of derivatives of N- $\alpha$ -acyl amino acids obtained from the condensation of a fatty

acid to the  $\alpha$ -amino group of the amino acid in the form of an ester. Among the most prominent derivatives are those of glutamic acid, sarcosine, lysine, and arginine ( ), which are N- $\alpha$ -acylates with fatty acids of chains comprising between 12 and 16 carbon atoms.

Until 1964 no data about the antimicrobial activity of synthetically obtained, noncyclic, amphiphilic peptides can be found in the literature. It was N. Molin who first presented the data of antimicrobial activity of the ethyl ester of N- $\alpha$ -palmitoyl-lys-lys, and later D. Kupryszewski, in 1976, confirmed the same results, associating the antimicrobial activity of these dipeptide derivatives with the following structural requisites:

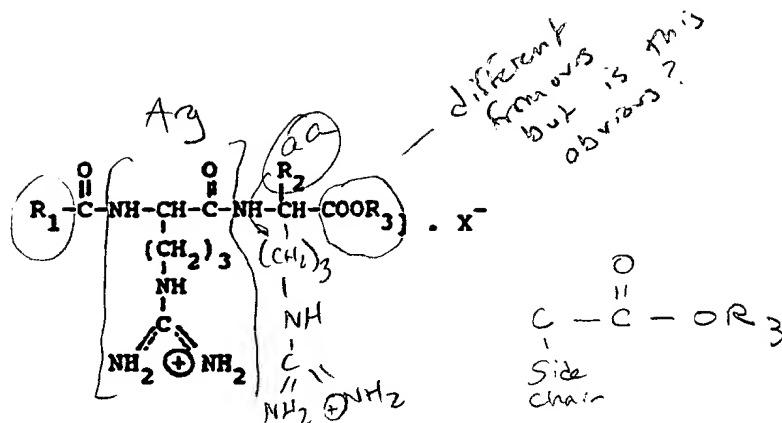
- terminal  $\alpha$ -amino group acylated with palmitic acid
- two free, protonated  $\omega$ -aminos
- lysine residues bonded by a peptide bond
- the esterified  $\alpha$ -carboxyl group.

In other words, the consequent surfactant-peptide base antimicrobial power would seem to be associated only with those amino acid and/or peptide derivatives that would have cationic surfactant characteristics.

In 1986 María R. Infante patented a procedure for the synthesis of dipeptides of fatty chain N-acyl arginine and protein hydrolyzates as antimicrobial ionic surfactants. The ionic nature of these surfactants could be cationic and/or amphoteric as a function of the chemical nature of the carboxyl group. These surfactants presented the advantage over previously published and/or patented [works], of being antimicrobial peptide-based surfactants of amphoteric nature, and hence less irritant than any other peptide-based cationic surfactant.

It must be pointed out that the molecules whose patent is applied for are similar to those patented by María R. Infante in 1986. The latter can chemically be considered as a polydisperse mixture of derivatives of arginine dipeptides acylated with a fatty acid, whereas those that we present as novel are derivatives of arginine acylated with fatty acid of a defined, homogeneous chemical structure. Their antimicrobial properties have been made possible while keeping their irritability indices very low, and in one case null.

The present invention is related to the preparation of lipopeptidic surfactants in which the hydrophilic part is formed by a dipeptide whose amino acid that carries the carbonyl group to the peptide bond is arginine, and the hydrophobic part consists of a fatty acid bonded to the arginine free amino group by an acyl bond according to the scheme of the formula.



Where

-  $R_1$  is a saturated or unsaturated hydrocarbon linear chain of 9 to 17 carbon atoms, which may contain hydroxy substituents.

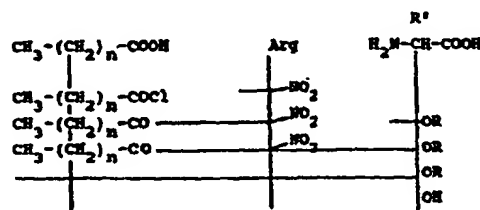
-  $R_2$  is the lateral chain of an amino acid, which may be one or any of the 20 natural amino acids; H- for glycine,  $\text{HO-CH}_2$ - for serine,  $\phi\text{-CH}_2$  for phenylalanine, etc.

-  $R_3$  is a short-chain alkyl residue or a monocation, including  $\text{H}^+$ .

-  $\text{X}^-$ :  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{CH}_3\text{-COO}^-$ .

The ionic nature of these molecules depends fundamentally on the  $R_3$  substitute. They will be cationic if  $R_3$  is an alkyl residue. They will be amphoteric if  $R_3$  is a monocation.

They are obtained by condensation of the fatty derivative of N- $\alpha$ -arginine and the alkyl ester (Me, Et, or Pro) of a pure amino acid, using for it the mixed anhydride condensation method as indicated in the following diagram:



The present invention encompasses, among other things, the preparation of the following compounds:

1. Nitroarginine.
2. N- $\alpha$ -decyl, N- $\alpha$ -dodecyl, N- $\alpha$ -tetradecyl, or N- $\alpha$ -hexadecyl nitroarginine.
3. Methyl, ethyl, or propyl esters of amino acid.
4. Alkyl esters of dipeptides of N- $\alpha$ -decyl, N- $\alpha$ -dodecyl, N- $\alpha$ -tetradecyl, or N- $\alpha$ -hexadecyl nitroarginine
5. Alkyl esters of dipeptides of N- $\alpha$ -decyl, N- $\alpha$ -dodecyl, N- $\alpha$ -tetradecyl, or N- $\alpha$ -hexadecyl arginine
6. Dipeptides of N- $\alpha$ -decyl, N- $\alpha$ -dodecyl, N- $\alpha$ -tetradecyl, or N- $\alpha$ -hexadecyl arginine.

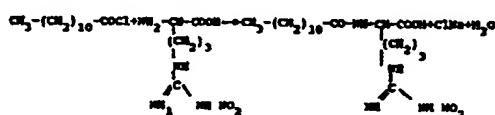
The importance of this synthesis lies fundamentally in the surfactant and antimicrobial properties of the final product in each case, as well as in the natural characteristics of its chemical structure [being] compatible with the protein structures of human skin.

By way of example, and without thereby limiting the procedure, we will describe in detail the synthesis of the following compounds.

1. Methyl ester chlorohydrate of N- $\alpha$ -lauroyl - arginyl - phenylalanine (cationic derivative)
2. Chlorohydrate of N- $\alpha$ - lauroyl - arginyl - phenylalanine (amphoteric derivative)

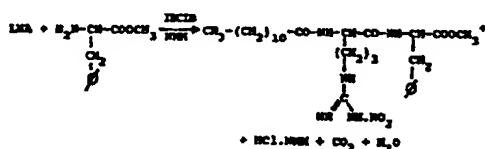
### Example 1

#### 1. Preparation of N-lauroyl-nitroarginine (LNA)



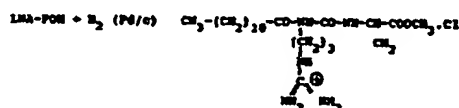
45 g of nitroarginine are suspended in a water/acetone mixture and the equivalent quantity of NaOH is added. The solution is cooled to  $-5^\circ\text{C}$  and 53.8 mL of lauroyl chloride and a slight excess of NaOH are added so that the pH is maintained between 11 and 13. When the addition is finished, the reaction mixture is left for a few hours at  $0^\circ\text{C}$  and later 1N HCl is added until the pH is acidic. A white solid appears, which is filtered and washed until the pH is neutral. It is dried and crystallized in ethanol/water. Yield: 60%, m.p.:  $174-178^\circ\text{C}$ ,  $[\alpha]_{20} = 4.4^\circ$  ( $c = 1$ , methanol).

#### 2. Preparation of the methyl ester of N-lauroyl nitro-arginyl-phenylalanine (LNA-POM)



Equimolecular quantities of LNA (8.5 g) and N-methyl morpholine (NMM) are dissolved in 50 mL of dry dimethylformamide (DMF). The mixture is cooled and 2.9 mL of isobutyl chloroformate (IBClF) are added slowly. The resulting mixture is energetically agitated, and immediately afterwards a solution containing the equimolecular quantity of the methyl ester of phenylalanine dissolved in 100 mL of DMF is added. The reaction mixture thus prepared is kept in agitation for 1 h in cold conditions, and for another 24 h at ambient temperature. The solvent is evaporated and the residue is washed with sodium bicarbonate at 5%, 0.1N HCl, and water. Thus LNA-POM is obtained with a 70% yield. m.p.:  $102-107^\circ\text{C}$ .

#### 3. Preparation of the methyl ester chlorohydrate of N-lauroyl-arginyl-phenylalanine (LA-POM)



Five grams of the previous solid are dissolved in 50 mL of formic acid and hydrogenated over 1 g of Pd/C for 6 h.

The unprotected derivative in the form of formic salt is purified by chromatography in a silica gel column using a mixture of methanol/chloroform in different proportions as an eluent. Thus 4 g of a very hygroscopic solid that crystallizes in HCl (MeOH)/ethyl ether, corresponding to LA POM in the form of chlorohydrate, are obtained.

The structure of the compound is determined by elemental analysis,  $^1\text{H}$ -NMR, EM-FAB, automatic amino acid analysis.

Determinations of fundamental surfactant, antimicrobial, and irritant properties (Draize test) indicated that this was a non-irritant, antimicrobial surfactant.

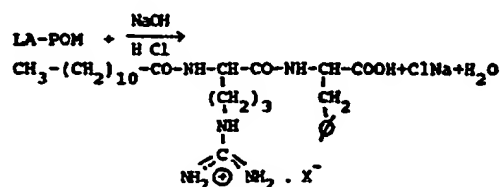
All data corresponding to the characteristics of the synthesis, characteristics of the compound, and its properties are indicated in Tables I and II.

### Example 2

Synthesis of the chlorohydrate of N- $\alpha$ -lauroyl-arginyl-phenylalanine (LA-POH)

The LA-POH derivative was obtained without noticeable differences by two different routes:

a) By direct saponification of LA-POM



One gram of LA POM, obtained according to the procedure described above, is dissolved in 10 mL of methanol and, in cold conditions, 3.2 mL of 1N NaOH are added. The mixture is kept in agitation at ambient temperature for 4 h, after which a white solid appears, which is repeatedly washed with water until it reaches neutral pH. This solid is dissolved in HCl (MeOH) in a molar ratio of 1/4 and ethyl ether is added until turbidity [forms]. A very hygroscopic solid precipitates, which is purified in a silica gel column and which corresponds, after the appropriate structural determinations, to the LA-POH object compound of the synthesis. After purification in a column, the yield of the reaction is 50%.

b) By saponification of LNA-POM and later hydrogenation.

2.25 g of LNA-POM are suspended in 9.91 mL of 0.5N NaOH. The solution is agitated for 3 h at 30°C. It is cooled and 1N HCl is added to pH 1.5-2. 2.15 g of a white solid appears, which is washed with water and dried, and which corresponds to the LNa-goh [sic; LNA-POH] derivative, which is hydrogenated as described in method a) above.

As for the previous compound, all data are indicated in Tables I and II.

Table I. Characteristics of the synthesized compounds.

① Compuesto	② Rtd.	③ Análisis Elemental		
LA POM peso molecular 553.5	60 %	⑤ % C teórico 58.79 esp. 58.85 ⑥	% H 8.75 8.85	%N 12.25 12.21

Key: 1 Compound  
2 Yield  
3 Elemental Analysis  
4 LA POM molecular weight  
5 Theor.  
6 Exp.

Table I (Continuation)

① Compuesto	② Rtd.	③ Análisis Elemental		
LA-POH peso molecular 539.5	a) 50 % b) 60 %	⑤ teórico 58.12 exp. 57.45 ⑥	8.61 8.74	12.56 12.86

Key: 1 Compound  
2 Yield  
3 Elemental Analysis  
4 LA-POH molecular weight  
5 Theor.  
6 Exp.



Table I (Continuation)

① Compuesto	② pf (° C)	④ [α] <sub>20°C</sub>
LA POM peso molecular 553.5	35-39°C	C= 1, metanol -1.68°C
LA-POH peso molecular 539.5	62-62°C	-1.60°C

Key: 1 Compound  
 2 m.p.  
 3 LA POM molecular weight  
 4 Methanol  
 5 LA-POH molecular weight

Table II. Stabilized surface tension (δ), critical micellar concentration (cmc), and minimal inhibitory concentration (MIC) of LA-POM and LA-POH.

① Compuesto	γ m New/m	cmc moles/l
LA POM	34.6	1.5x10 <sup>-3</sup>
LA-POH	30.0	0.25x10 <sup>-3</sup>

Key: 1 Compound

Table II (Continuation)

① Compuesto	MIC (g/ml)					
	1	2	3	4	5	6
LA POM	16	4	4	16	16	256

Key: 1 Compound

Table II (Continuation)

① Compuesto	MIC (g/ml)					
	1	2	3	4	5	6
LA.POH	4	4	2	4	4	128

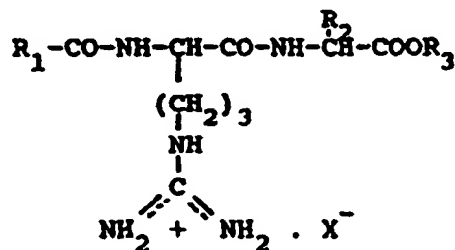
Key: 1 Compound

- 1: *Candida albicans*, CCM(1);  
 2: *Staphylococcus epidermidis* ATCC 12228;  
 3: *Micrococcus aurantiacus* ATCC 11731;  
 4: *Bacillus subtilis* ATCC6623  
 5: *Escherichia coli* ATCC;  
 6: *Pseudomonas aeruginosa* ATCC 10145.

(1) Chair of Microbiology. School of Pharmacy of Barcelona.

### Claims

1. "Procedure for the synthesis of dipeptides of fatty chain N-acyl arginine and (acidic, basic, or neutral) pure amino acids as non-irritant ionic surfactants of antimicrobial action" of the general formula:



where

-  $R_1$  is a saturated or unsaturated hydrocarbon linear chain of 9 to 17 carbon atoms, which may contain hydroxy substituents.

-  $R_2$  is the lateral chain of amino acid, which may be any one of the 20 natural amino acids:

H- for glycine,  $HO-CH_2-$  for serine,  $\phi-CH_2$  for phenylalanine, etc.

-  $R_3$  is a short-chain alkyl residue or a monocation, including  $H^+$

-  $X^-$ :  $Cl^-$ ,  $Pr^-$  [sic;  $Br^-$ ],  $CH_3-COO^-$

characterized in that, in a first stage, the chloride of fatty acid condenses to the derivative of nitroarginine amino acid; in a second stage the dipeptide is formed by condensation of a second amino acid in the form of ester to the compound fatty chain N-acyl nitroarginine, and finally, in a third stage, the nitro group of the N- $\alpha$ -acyl nitro arginyl-amino acid is fundamentally reduced by catalytic hydrogenation and/or saponification of the ester group of the terminal amino acid.

2. A procedure according to Claim 1, characterized in that it uses L-Arg, D-Arg, or DL-Arg as the amino acid of departure.

3. A procedure according to Claim 1, characterized in that it uses the nitro group as a protector of the arginine guanidine group.

4. A procedure according to Claim 1, characterized in that it uses saturated, unsaturated, or hydroxy substituted linear fatty acid chlorides, pure or [in a] mixture, of 10 to 18 carbon atoms, to acylate the nitroarginine in a water/alcohol medium of pH: 9-10.

5. A procedure according to Claim 1, characterized in that it uses short-chain alkyl esters of pure amino acids to form the dipeptides of fatty chain N- $\alpha$ -acyl arginine forming the object of the present patent.

6. A procedure according to Claim 1, characterized [in that] the condensation of N- $\alpha$ -acyl nitroarginine with the ester of the pure amino acid, to obtain the N- $\alpha$ -acyl nitro arginyl-amino acid, takes place through the formation of a mixed anhydride as follows: for 90 sec and in cold conditions, isobutyl chloroformate, N-methyl morpholine, and N- $\alpha$ -acyl nitroarginine are mixed equimolecularly in the presence of dimethylformamide; then the ester of the amino acid previously neutralized with N-methyl morpholine is added to this mixture, and it is left in agitation for a maximum of 24 h at ambient temperature.

7. A procedure according to Claims 1 and 6, characterized in that the purification of the dipeptides of N-acyl nitroarginine is carried out easily through organic and aqueous extractions.

8. A procedure according to Claim 1, characterized in that the deprotection of the nitro group for obtaining the amphiphilic peptides forming the object of the present patent, takes place with hydrogen gas in a medium containing Pd/C and formic acid.

9. A procedure according to Claim 1, characterized in that the deprotection of the esterified derivatives to form the derivatives with a free terminal carboxyl group takes place through direct saponification of the esters of N- $\alpha$ -acyl-arg-amino acids in an alcoholic medium containing NaOH.

10. A procedure according to Claim 1, characterized in that the extreme purification of the products takes place by column chromatography, using a gradient system of

Procedure for obtaining dipeptides of fatty chain N- $\alpha$ -acyl arginine and pure amino acids as water-soluble, biodegradable, non-irritant, ionic surfactants with antimicrobial action.